METHOD OF REDUCING THE HARMFUL EFFECTS OF ORALLY OR TRANSDERMALLY DELIVERED NICOTINE

FIELD OF THE INVENTION

The present invention generally relates to the reduction of the harmful effects of orally or transdermally delivered nicotine in conventional tobacco-use cessation programs. More specifically, embodiments concern methods of reducing the harmful effects of nicotine intake, associated with conventional tobacco-use cessation programs, by providing tobacco products, which contain a reduced amount of nicotine and tobacco specific nitrosamines (TSNAs).

BACKGROUND OF THE INVENTION

The addictive properties of tobacco products are largely attributable to the presence of nicotine and the habitual use of the delivery system (e.g., the oral fixation associated with the act of smoking or chewing tobacco, smoke intake, and taste). Many tobacco-use cessation programs involve the use of nicotine replacement therapy (NRT), wherein various amounts of nicotine are given to the individual as a replacement for tobacco use. Several types of tobacco-use cessation products, which involve NRT, are currently available. For example, nicotine patches, gums, capsules, inhalers, nasal sprays, and lozenges are conventional products of NRT. Although these conventional products of NRT may help tobacco users by suppressing the symptoms of nicotine withdrawal, they do little to satisfy a tobacco user's cravings for the habitual use of the delivery system. (Dotinga, *Study Bursts Nicotine Gum's Bubble,* Health - Health Scout News, September 20, 2002). The factors involved with the habitual use of the delivery system are hereinafter referred to as "secondary factors of addiction." These secondary factors of addiction are largely psychological factors that have only an incidental relationship to the chemical dependence on nicotine.

In addition to the fact that conventional NRT does little to quell the secondary factors of addiction, NRT has had only limited success in enabling people to quit tobacco use. For example, among over-the-counter NRT gum users, abstinence rates were 16.1% at 6 weeks and 8.4% at 6 months; whereas, for prescription NRT gum users abstinence rates were 7.7% at 6 weeks and 7.7% at 6 months. (Shiffman et al., *Addiction* 97:505-516, 2002). Users of the of the NRT patch experienced only slightly better results; over-the

counter patch users were reported to have 19.0% abstinence at 6 weeks and 9.2% at 6 months; whereas, prescription NRT patch users experienced 16.0% abstinence at 6 weeks and 3.0% abstinence at 6 months. *Id.* Others report slightly better results in that smoking cessation with patch or gum show verified abstinence rates at 12 months in the range of 20%. (O'Brien, *Lecture given to medical students at the University of Pennsylvania on September 22, 1995*). One study, however, goes so far as to say that NRT is no longer effective in increasing long-term successful cessation in California smokers. (Pierce and Gilpin, *Jama*, 288:1260-1264 (2002)). Clearly, it appears that tobacco addiction is a complex web of psychological factors (i.e., the secondary factors) coupled with nicotine dependence and existing NRT is largely ineffective.

By design, conventional NRT relies on tobacco users to gradually reduce their daily nicotine intake, while they mentally curb their cravings for the secondary factors of addiction. In practice, however, many program participants only replace the addiction for tobacco with a far more expensive addiction to the NRT product. In some cases, program participants ingest far more nicotine than they would from conventional tobacco use to compensate for lack of fulfillment of the secondary factors of addiction. In other cases, program participants continue using the NRT product for long periods after the initial program has been completed and eventually return to tobacco products.

The intake of large amounts of nicotine and long-term use of NRT raises serious health concerns. In some cases, nicotine overdose may occur with overzealous use of NRT products. Symptoms of nicotine overdose include nausea and/or vomiting, increased watering of mouth (severe), abdominal or stomach pain (severe), diarrhea (severe), pale skin, cold sweat, headache (severe), dizziness (severe), disturbed hearing and vision, tremor, confusion, weakness (severe), extreme exhaustion, fainting, low blood pressure, difficulty in breathing (severe), irregular heartbeat, or convulsions (seizures). Psychological stress may also occur in individuals using NRT for long periods of time because nicotine releases epinephrine, a hormone that stimulates a stress response in the body. The psychological effects of nicotine include irritability, anxiety, sleep disturbances, nervousness, poor mood and temperament, headaches, fatigue, nausea, and a long-term craving for tobacco.

Furthermore, recent research has established that nicotine stimulates the growth of blood vessels during periods of inflammation and promotes angiogenesis, atherosclerosis and tumor growth. (Heeschen, *et al.*, *Nature Medicine* 7:833, 2001). Nicotine may also be a precursor for the endogenous formation of carcinogenic substances such as 4-

(methynitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by the body's own metabolic system. (Hecht *et al.*, *Proc. Nat. Acad. Sci.* 97:12493-12497, 2000). There remains a need for nicotine reduction and/or tobacco-use cessation programs that utilize tobacco products that that contain reduced amounts of nicotine and TSNAs.

SUMMARY OF THE INVENTION

The present invention relates to methods of reducing the harmful effects of nicotine intake associated with conventional tobacco-use cessation programs. More specifically, tobacco products with reduced levels of tobacco specific nitrosamines (TSNAs) are provided. Additionally, methods of making or blending these reduced TSNA tobacco products are provided.

In some embodiments of the present invention, methods of making a blended reduced nicotine tobacco are provided, by providing a first tobacco; a second tobacco produced from a genetically modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety); and blending the first and second tobacco to obtain a reduced nicotine tobacco. A tobacco product having the blended reduced nicotine tobacco produced by this method is also provided.

In further embodiments, methods of making a blended reduced TSNA tobacco are provided, by providing a first tobacco; a second tobacco produced from a genetically modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety); and blending the first and second tobacco to obtain the reduced TSNA tobacco. A tobacco product having the blended reduced TSNA tobacco produced by this method is also provided.

In yet further embodiments, methods of making a reduced nicotine tobacco product with a desired amount of nicotine are provided, by providing a first tobacco which has a measured amount of nicotine; a second tobacco with a measured amount of nicotine, produced from a genetically modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety); and blending the first and second tobacco to produce a reduced nicotine tobacco product with a desired amount of nicotine. The reduced nicotine tobacco product can be, for example, a blended cigarette. The blended cigarette can contain, for example, 0.6 mg or less nicotine, 0.3 mg or less nicotine, or 0.05 mg or less nicotine. Tobacco products having the blended reduced nicotine tobacco produced by these methods are also provided. Additionally, tobacco-use cessation kits having tobacco products produced by these methods are provided.

Additional embodiments of the present invention provide methods of making a reduced TSNA tobacco product with a desired amount of TSNA by providing a first tobacco with a measured amount of TSNA; providing a second tobacco with a measured amount of TSNA, produced from a genetically modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety); and blending the first and second tobacco to produce a reduced TSNA tobacco product with a desired amount of TSNA.

Further embodiments of the present invention provide methods of reducing the nicotine consumption of a tobacco user by providing to the tobacco user a first tobacco product having tobacco produced from a modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety); and a second tobacco product having tobacco produced from a modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety), where the second tobacco product has less nicotine than the first tobacco product; providing the tobacco user with additional tobacco products having tobacco produced from a modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety), where subsequent tobacco products have sequentially reduced amounts of nicotine, starting with a third product that has less nicotine than the first or second tobacco product.

Yet further embodiments of the present invention provide methods of reducing the TSNA consumption of a tobacco user by providing the tobacco user with a first tobacco product having tobacco produced from a modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety); and a second tobacco product having tobacco produced from a modified tobacco plant having a reduced level of QPTase (as compared to an unmodified tobacco plant of the same variety), where the second tobacco product has less TSNA than the first tobacco product; and further providing the tobacco user with additional tobacco products having tobacco produced from a modified tobacco plant having a reduced level of QPTase, as compared to an unmodified tobacco plant of the same variety, wherein the subsequent tobacco products have sequentially reduced amounts of TSNA, starting with a third product that has less TSNA than the first or second tobacco product.

Additional embodiments of the present invention provide for the use of a genetically modified tobacco produced from a tobacco plant having a reduced amount of QTPase to prepare a blended tobacco product that has a selected amount of nicotine. The

blended tobacco product can be, for example, a blended cigarette. The blended tobacco product can be, for example, a blended cigarette having 0.6 mg nicotine or less, 0.3 mg nicotine or less, or 0.05 mg nicotine or less.

Further embodiments of the present invention provide for the use of a genetically modified tobacco produced from a tobacco plant that has a reduced amount of QTPase to prepare a blended tobacco product that has a selected amount of TSNA. The blended tobacco product can be, for example, a blended cigarette. The blended tobacco product can be, for example, a blended cigarette having 0.6 mg nicotine or less, 0.3 mg nicotine or less, or 0.05 mg nicotine or less.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns nicotine reduction and/or tobacco-use cessation programs, which involve the use of modified tobacco products that contain reduced amounts of nicotine and TSNAs. While most tobacco cessation programs rely heavily on nicotine replacement therapy (NRT), many of the embodiments described herein focus less on nicotine replacement and more on replacing the secondary factors of addiction such as smoke intake, oral fixation, and taste. A copending application entitled "Modifying Nicotine and Nitrosamine levels in Tobacco" (WO02100199), which was published in English designating the United States of America and claiming priority to U.S. Provisional Application No. 60/371635. Also incorporated by reference in their entireties are related U.S. Patent Nos. 6,586,661 and 6,423,520.

Some embodiments of the invention concern the use of low nicotine and TSNA tobacco products that have burning and taste characteristics that are virtually indistinguishable from conventional tobacco products. While there are many ways to create such reduced nicotine and/or TSNA products, the preferred methods use techniques in plant genetic engineering to reduce or eliminate enzymes involved in nicotine biosynthesis. Preferably, techniques in plant genetic engineering are used to selectively reduce the amount of the enzyme quinolate phosphoribosyl transferase (QPTase), which is involved in the production of nicotine at the root cortex. There may be many ways to reduce levels of QPTase in tobacco plants, given the teachings described herein and the level of skill in the art, however, the preferred methods involve the use of antisense technology or molecular decoy technology.

Several approaches to create tobacco and tobacco products that have a reduced amount of nicotine and/or TSNAs have been discovered. Interestingly, it was discovered

that TSNA content in a tobacco plant can be lowered by reducing the nicotine content in the tobacco plant. In some embodiments, antisense technology is used to lower nicotine and TSNA levels in tobacco plants. (*See* PCT/US98/11893). In other embodiments, molecular decoy technology is used to lower nicotine and/or TSNA levels in tobacco plants (See U.S. patent application serial number 09/941,042).

By one approach, for example, a DNA construct encoding an antisense RNA that complements at least a portion of the QPTase gene **(SEQ. ID. No. 1)** is prepared such that transcription of the complementary strand of RNA reduces expression of the endogenous quinolate phosphoribosyl gene, which, in turn, reduces the amount of nicotine and, concomitantly, the amount of TSNA in the tobacco plant. By another approach, transcription factor molecular decoys for the QPTase gene, which are nucleic acid fragments that correspond to the 5' upstream regulatory elements (e.g., Nic 1 and Nic 2 transcription factor binding sites) are inserted into the plant cell. The transcription factors bind to the decoy fragments rather than the endogenous transcription factor binding sites and a reduction in the level of transcription of QPTase is obtained.

Once the transgenic tobacco plants having reduced nicotine and/or TSNA are made, the tobacco is harvested and cured by conventional methods and is incorporated into a variety of tobacco products. Preferably, the transgenic tobacco is blended such that specific amounts of nicotine and/or TSNA are obtained in specific products. That is, the blending is conducted so that tobacco products of varying amounts of nicotine and/or TSNAs are made. In this manner, a step-wise tobacco-use cessation program can be established, wherein a program participant begins the program at step 1 with a tobacco product having only slightly less nicotine; at step 2 the program participant begins using a tobacco product with less nicotine than the products used in step 1; at step 3, the program participant begins using a tobacco product with less nicotine than the products in step 2; and so on, for as many steps as desired for a particular tobacco-use cessation program. Ultimately, the tobacco product used by the program participant can have an amount of nicotine that is less than that which is required to become addictive or maintain an addiction. In this manner, a nicotine reduction and/or tobacco-use cessation program is provided that limits the exposure of a program participant to nicotine yet retains the secondary factors of addiction, including but not limited to, smoke intake, oral fixation, and taste. The following section describes tobacco products that can be used with the tobacco-use cessation programs described herein.

Tobacco products for use in nicotine reduction and/or tobacco-use cessation programs

Wild type tobacco varies significantly in the amount of TSNAs and nicotine depending on the variety and the manner it is grown, harvested, and cured. For example, a typical Burley tobacco leaf can have about 30,000 parts per million (ppm) nicotine and 8,000 parts per billion (ppb) TSNA; a typical Flue-Cured Burley leaf can have about 20,000 ppm nicotine and 300 ppb TSNA; and a typical Oriental cured leaf can have about 10,000 ppm nicotine and 100 ppb TSNA. A tobacco plant or portion thereof having a reduced amount of nicotine and/or TSNA, for use with aspects of the invention, can have no detectable nicotine and/or TSNA, or may contain some detectable amounts of one or more TSNA and/or nicotine so long as the amount of nicotine and/or TSNA is less than that found in a control plant of the same variety.

That is, a Burley tobacco leaf embodiment of the invention having a reduced amount of nicotine can have between about 0 and about 30,000 ppm nicotine and about 0 and about 8,000 ppb TSNA desirably, between about 0 and about 20,000 ppm nicotine and about 0 and about 6,000 ppb TSNA more desirably, between about 0 and about 10,000 ppm nicotine and about 0 and about 5,000 ppb TSNA preferably, between about 0 and about 5,000 ppm nicotine and about 0 and about 4,000 ppb TSNA more preferably, between about 0 and about 2,500 ppm nicotine and about 0 and about 2,000 ppb TSNA even more preferably, and most preferably between about 0 and about 1,000 ppm nicotine and about 0 and about 1,000 ppb TSNA. Embodiments of Burley leaf prepared by the methods described herein can also have between about 0 and about 1000 ppm nicotine and about 0 and about 500 ppb TSNA and some embodiments of Burley leaf prepared by the methods described herein have virtually no detectable amount of nicotine or TSNA.

Similarly, a Flue-cured tobacco leaf for use with the disclosed methods can have a reduced amount of nicotine which is between about 0 and about 20,000 ppm nicotine and about 0 and about 300 ppb TSNA desirably between about 0 and about 15,000 ppm nicotine and about 0 and about 250 ppb TSNA more desirably between about 0 and about 10,000 ppm nicotine and about 0 and about 200 ppb TSNA preferably between about 0 and about 5,000 ppm nicotine and about 0 and about 150 ppb TSNA more preferably between about 0 and about 2,500 ppm nicotine and about 0 and about 100 ppb TSNA and most preferably between about 0 and about 1,000 ppm nicotine and about 0 and about 50 ppb TSNA. Embodiments of flue-cured tobacco prepared by the methods described herein can also have between about 0 and about 500 ppm nicotine and about 0 and about 25 ppb

TSNA and some embodiments of flue-cured tobacco prepared by the methods described herein have virtually no detectable amount of nicotine or TSNA.

Further, an Oriental cured tobacco for use with the embodied methods can have a reduced amount of nicotine having between about 0 and about 10,000 ppm nicotine and about 0 and about 100 ppb TSNA desirably between about 0 and about 7,000 ppm nicotine and about 0 and about 75 ppb TSNA more desirably between about 0 and about 5,000 ppm nicotine and about 0 and about 50 ppb TSNA preferably between about 0 and about 3,000 ppm nicotine and about 0 and about 25 ppb TSNA more preferably between about 0 and about 1,500 ppm nicotine and about 0 and about 10 ppb TSNA and most preferably between about 0 and about 500 ppm nicotine and essentially no TSNA. Embodiments of Oriental cured tobacco prepared by the methods described herein can also have between about 0 and about 250 ppm nicotine and essentially no TSNA and some embodiments of Oriental cured tobacco prepared by the methods described herein have virtually no detectable amount of nicotine or TSNA.

As discussed above, TSNAs and nicotine contribute significantly to the carcinogenic potential and addictive properties of tobacco and tobacco products. Thus, tobacco and tobacco products that have a reduced amount of TSNA and nicotine have tremendous utility. It was found that the reduction of nicotine in tobacco was directly related to the reduction of TSNAs. Unexpectedly, the methods described herein not only produce tobacco with a reduced addictive potential but, concomitantly, produce a tobacco that has a lower carcinogenic potential.

It should be emphasized that the phrase "a reduced amount" is intended to refer to an amount of nicotine and or TSNA in a treated or transgenic tobacco plant, tobacco, or a tobacco product that is less than what would be found in a tobacco plant, tobacco, or a tobacco product from the same variety of tobacco processed in the same manner, which has not been treated or was not made transgenic for reduced nicotine and/or TSNA. Thus, in some contexts, wild-type tobacco of the same variety that has been processed in the same manner is used as a control by which to measure whether a reduction in nicotine and/or TSNA has been obtained.

In some contexts, the phrase reduced amount of nicotine and/or TSNAs refers to the tobacco plants, tobacco and tobacco products of the invention that have less nicotine and/or TSNAs by weight than the same variety of tobacco grown, processed, and cured in the same way. For example, wild type tobacco can contain approximately 1-4% dry weight nicotine and approximately 0.2% - 0.8% dry weight TSNAs depending on the variety, and

the manner in which it was grown, harvested and cured. A typical cigarette has 11 mg of nicotine and 8μg of TSNAs. Thus, the tobacco plants, tobacco and tobacco products of the invention can have, in dry weight for example, less than 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.055%, 0.06%, 0.065%, 0.07%, 0.075%, 0.08%, 0.085%, 0.09%, 0.095%, 0.1%, 0.15%, 0.175%, 0.2%, 0.225%, 0.25%, 0.275%, 0.3%, 0.325%, 0.35%, 0.375%, 0.4%, 0.425%, 0.45%, 0.475%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, and 1.0% nicotine and less than 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.055%, 0.06%, 0.065%, 0.07%, 0.075%, and 0.08% TSNA.

Alternatively, a tobacco product, e.g., a cigarette, can have, for example, less than 0.01mg, 0.05mg, 0.1mg, 0.15mg, 0.2mg, 0.25mg, 0.3mg, 0.35mg, 0.4mg, 0.45mg, 0.5mg, 0.55mg, 0.6mg, 0.65mg, 0.7mg, 0.75mg, 0.8mg, 0.85mg, 0.9mg, 0.95mg, 1.0mg, 1.1mg, 1.15mg, 1.2mg, 1.25mg, 1.3mg, 1.35mg, 1.4mg, 1.45mg, 1.5mg, 1.55mg, 1.6mg, 1.65mg, 1.7mg, 1.75mg, 1.8mg, 1.85mg, 1.9mg, 1.95mg, 2.0mg, 2.1mg, 2.15mg, 2.2mg, 2.25mg, 2.3mg, 2.35mg, 2.4mg, 2.45mg, 2.5mg, 2.55mg, 2.6mg, 2.65mg, 2.7mg, 2.75mg, 2.8mg, 2.85mg, 2.9mg, 2.95mg, 3.0mg, 3.1mg, 3.15mg, 3.2mg, 3.25mg, 3.3mg, 3.35mg, 3.4mg, 3.45mg, 3.5mg, 3.55mg, 3.6mg, 3.65mg, 3.7mg, 3.75mg, 3.8mg, 3.85mg, 3.9mg, 3.95mg, 4.0mg,. 4.1mg, 4.15mg, 4.2mg, 4.25mg, 4.3mg, 4.35mg, 4.4mg, 4.45mg, 4.4mg, 4.45mg, 4.5mg, 4.55mg, 4.6mg, 4.65mg, 4.7mg, 4.75mg, 4.8mg, 4.85mg, 4.9mg, 4.95mg, 5.0mg, 5.5mg, 5.7mg, 6.0mg, 6.5mg, 6.7mg, 7.0mg, 7.5mg, 7.7mg, 8.0mg, 8.5mg, 8.7mg, 9.0mg, 9.5mg, 9.7mg, 10.0mg, 10.5mg, 10.7mg, and 11.0mg nicotine and less than 0.1 micrograms, 0.15 micrograms, 0.2 micrograms, 0.25 micrograms, 0.3 micrograms, 0.35 micrograms, 0.4 micrograms, 0.45 micrograms, 0.5 micrograms, 0.55 micrograms, 0.6 micrograms, 0.65 micrograms, 0.7 micrograms, 0.75 micrograms, 0.8 micrograms, 0.85 micrograms, 0.9 micrograms, 0.95 micrograms, 1.0 micrograms, 1.1 micrograms, 1.15 micrograms, 1.2 micrograms, 1.25 micrograms, 1.3 micrograms, 1.35 micrograms, 1.4 micrograms, 1.45 micrograms, 1.5 micrograms, 1.55 micrograms, 1.6 micrograms, 1.65 micrograms, 1.7 micrograms, 1.75 micrograms, 1.8 micrograms, 1.85 micrograms, 1.9 micrograms, 1.95 micrograms, 2.0 micrograms, 2.1 micrograms, 2.15 micrograms, 2.2 micrograms TSNA.

Several methods for reducing endogenous levels of nicotine and TSNAs in a plant have been discovered. These approaches can be used to create the tobacco products described above. Tobacco plants having a reduced amount of nicotine and/or TSNAs that

retain good smoking characteristics and taste, as manufactured by the methods described in the following section, can be used in the embodied tobacco-use cessation programs.

Approaches to make tobacco products having reduced nicotine and/or TSNA levels

Nicotine is produced in tobacco plants by the condensation of nicotinic acid and 4-methylaminobutanal. Two regulatory loci (*Nic1* and *Nic2*) act as co-dominant regulators of nicotine production. These two loci are unlinked and the gene action is semi-dominant and primarily additive (Legg *et al.* (1969) *J. Hered.*, 60, 213-217).

Genetic and enzyme analyses have been used to investigate the *Nic1* and *Nic2* genes. Collins *et al.* ((1974) *Crop Sci.*, 14, 77-80) prepared doubled haploid tobacco breeding lines of these four alkaloid genotypes. The genotype of standard cultivars is *Nic1/Nic1 Nic2/Nic2* and that of low nicotine lines is *nic1/nic1 nic2/nic2*. *Nic1/Nic1 nic2/nic2* is a high intermediate and *nic1/nic1 Nic2/Nic2* is a low intermediate (Legg and Collins (1971) *Can. J. Genet. Cytol.* 13, 287-291). These lines are similar in days-to-flower, number of leaves, leaf size, and plant height. Enzyme analyses of roots of single and double *Nic* mutants show that the activities of two enzymes, quinolate phosphoribosyl transferase (QPTase) and putrescine methyl transferase (PMTase), are directly proportional to levels of nicotine biosynthesis (Saunders and Bush (1979) *Plant Physiol* 64:236). Both *Nic1* and *Nic2* affect PMTase and QPTase activities in roots, and thus, regulate nicotine synthesis (Leete (1983) *In: Alkaloids: Chemical and Biological Perspectives*, S.W. Pelletier, ed. John Wiley & Sons, pp. 85-152).

Enzyme analyses of roots of single and double *Nic* mutants show that the activities of QPTase and PMTase are directly proportional to levels of nicotine biosynthesis. An obligatory step in nicotine biosynthesis is the formation of nicotinic acid from quinolinic acid, which step is catalyzed by QPTase. QPTase appears to be a rate-limiting enzyme in the pathway supplying nicotinic acid for nicotine synthesis in tobacco (See, eg., Feth *et al.*, *Planta*, 168, pp. 402-07 (1986) and Wagner *et al.*, *Physiol. Plant.*, 68, pp. 667-72 (1986)). A comparison of enzyme activity in tobacco tissues (root and callus) with different capacities for nicotine synthesis shows that QPTase activity is strictly correlated with nicotine content (Wagner and Wagner, *Planta* 165:532 (1985)). In fact, Saunders and Bush (*Plant Physiol* 64:236 (1979) showed that the level of QPTase in the roots of low nicotine mutants is proportional to the levels of nicotine in the leaves.

Hibi *et al.* ((1994) *Plant Cell*, 6, 723-735) isolated the cDNA encoding PMTase, *PMT*, and showed that *PMT* transcript levels are regulated by *Nic1* and *Nic2*. The QPTase cDNA and genomic clones (*NtQPT1*) have also been isolated and the transcript levels of *NtQPT1* are also regulated by *Nic1* and *Nic2*. Thus, it appears that the *Nic* genes regulate nicotine content by regulating the transcript levels of genes encoding the two rate-limiting enzymes, PMTase and QPTase. Further, *Nic1* and *Nic2* have been shown to be positive regulators of *NtQPT1* transcription and that promoter sequences upstream of the transcription initiation site contain the *cis*-acting sequences necessary for *Nic* gene product activation of *NtQPT1* transcription. Because expression of QPTase and PMTase are coordinately-regulated by the *Nic* gene products, it likely that the *Nic* gene products also directly regulate transcription of the PMT gene.

One approach for reducing nicotine involves reducing the amount of a required enzyme (e.g., QPTase and PMTase) in the biosynthetic pathway leading to its production. Where the affected enzyme naturally occurs in a rate-limiting amount (relative to the other enzymes required in the pathway), any reduction in that enzyme's abundance will decrease the production of the end product. If the amount of the enzyme is not normally rate-limiting, its presence in a cell must be reduced to rate-limiting levels in order to diminish the pathway's output. Conversely, if the naturally-occurring amount of enzyme is rate limiting, then any increase in the enzyme's activity will result in an increase in the biosynthetic pathway's end product.

The modification of nicotine levels in tobacco plants by antisense regulation of PMTase expression is proposed in US Patents 5,369,023 and 5,260,205 to Nakatani and Malik. PCT application WO 94/28142 to Wahad and Malik describes DNA encoding PMT and the use of sense and antisense PMT constructs. Additionally, PCT Application WO98/56923 to Conkling et al. describes DNA encoding a plant QPTase enzyme, constructs comprising such DNA, and methods of altering QPTase expression to increase or decrease nicotine production in tobacco plants. Still further, U.S. patent application serial number 09/941,042 to Conkling describes the use of DNA encoding regulatory sequences for the QPTase enzyme and methods of using these sequences as molecular decoys to sequester transcription factors at sites distant to the endogenous promoter for the QPTase gene, thereby decreasing nicotine production in tobacco plants. The following section describes in greater detail the antisense approach to making tobacco products having a reduced nicotine and/or TSNA level.

Antisense technology can be used to create tobacco products having a reduced level of nicotine and/or TSNA

Antisense technology may be used to create tobacco plants with reduced nicotine levels. The preferred enzyme for antisense regulation of nicotine levels is the *TobRD2* gene (see Conkling *et al.*, *Plant Phys.* 93, 1203 (1990)) encoding a *Nicotiana tabacum* QPTase (see **Example 1) (SEQ. ID. No. 1)**. In addition to the description of the technology provided herein, general aspects of the technology are described in PCT/US98/11893.

Regulation of gene expression in plant cell genomes can be achieved by integration of heterologous DNA under the transcriptional control of a promoter which is functional in the host, and in which the transcribed strand of heterologous DNA is complementary to the strand of DNA that is transcribed from the endogenous gene to be regulated. The introduced DNA, referred to as antisense DNA, provides an RNA sequence which is complementary to naturally produced (endogenous) mRNAs and which inhibits expression of the endogenous mRNA. Although the mechanism of antisense is not completely understood, it is known that antisense constructs can be used to regulate gene expression. A preferred approach for reducing QPTase levels through molecular modification is provided in **Example 2** and **Example 3**.

In some methods of the invention, the antisense product may be complementary to coding or non-coding (or both) portions of naturally occurring target RNA. The antisense construct may be introduced into the plant cells in any suitable manner, and may be integrated into the plant genome for inducible or constitutive transcription of the antisense sequence. Tobacco plants are then regenerated from successfully transformed cells using conventional techniques. It is most preferred that the antisense sequence utilized be complementary to the endogenous sequence, however, minor variations in the exogenous and endogenous sequences may be tolerated. It is preferred that the antisense DNA sequence be of sufficient sequence similarity that it is capable of binding to the endogenous sequence in the cell to be regulated, under stringent conditions as described below.

Although the preferred enzyme for antisense regulation is QPTase, other enzymes that are suitable for antisense regulation include, for example, putrescine N-methyltransferase, N-methylputrescine oxidase, ornithine decarboxylase, S-adenosylmethionine synthetase, NADH dehydrogenase, phosphoribosylanthranilate isomerase, and any other enzyme linked to nicotine biosynthesis.

As an example of the use of antisense technology, tobacco having a reduced amount of nicotine and TSNA is generated from a tobacco plant that is created by exposing at least one tobacco cell of a selected tobacco variety (preferably Burley 21) to an exogenous DNA construct having, in the 5' to 3' direction, a promoter operable in a plant cell and DNA containing a portion of a DNA sequence that encodes an enzyme in the nicotine synthesis pathway or a complement thereof (e.g., **SEQ. ID. No. 1)**. The DNA is operably associated with said promoter and the tobacco cell is transformed with the DNA construct. The transformed cells are selected using either negative selection or positive selection techniques and at least one tobacco plant is regenerated from transformed cells. The regenerated tobacco plant or portion thereof is preferably analyzed to determine the amount of nicotine and/or TSNA present and these values can be compared to the amount of nicotine and/or TSNA present in a control tobacco plant or portion, preferably of the same variety.

The DNA constructs having a portion of a DNA sequence that encodes an enzyme in the nicotine synthesis pathway may have the entire coding sequence of the enzyme a complement of this sequence, or any portion thereof. A portion of a DNA sequence that encodes an enzyme in the nicotine synthesis pathway or the complement thereof may have at least 25, 27, 30, 35, 40, 45, 50, 60, 75, 100, 150, 250, 500, 750, 1000, 1500, 2000, 2500, or 5000 bases, or the entire coding sequence of the enzyme or complement thereof (e.g., **SEQ. ID. No. 1)**. Accordingly, these DNA constructs have the ability to perturb the production of endogenous enzyme in the nicotine biosynthesis pathway through either an antisense or cosuppression mechanism. It is contemplated that both antisense, RNAi, and cosuppression constructs are effective at reducing the levels of nicotine and/or nitrosamines in tobacco plants.

Nucleic acid sequences employed in the constructs described herein include those with sequence similarity to the gene encoding QPTase, and encoding a protein having quinolate phosphoribosyl transferase activity, including, for example, allelic variations in QPTase proteins. Thus, DNA sequences that hybridize to DNA of the QPTase-encoding gene and code for expression of QPTase, particularly plant QPTase enzymes, may also be employed in carrying out the present invention. Multiple forms of tobacco QPT enzyme may exist. Multiple forms of an enzyme may be due to post-translational modification of a single gene product, or to multiple forms of the NtQPT1 gene.

As used herein, the term 'gene' can refer to a DNA sequence that incorporates (1) upstream (5') regulatory signals including the promoter, (2) a coding region specifying the

product, protein or RNA of the gene, (3) downstream regions including transcription termination and polyadenylation signals and (4) associated sequences required for efficient and specific expression. In some contexts, a gene can include only (2), above, or some combination of items (1), (3), and (4) with (2). The DNA sequence of the present invention may comprise or consist essentially of the sequence encoding the QPTase enzyme, or equivalent nucleotide sequences representing alleles or polymorphic variants of these genes, or coding regions thereof. Use of the phrase "substantial sequence similarity" in the present specification and claims means that DNA, RNA or amino acid sequences which have slight and non-consequential sequence variations from the actual sequences disclosed and claimed herein are considered to be equivalent to the sequences of the present invention. In this regard, "slight and non-consequential sequence variations" mean that "similar" sequences (i.e., the sequences that have substantial sequence similarity with the DNA, RNA, or proteins disclosed and claimed herein) will be functionally equivalent to the sequences disclosed and claimed in the present invention. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein.

By one approach, a novel cDNA sequence encoding a plant QPTase may be used. As QPTase activity is strictly correlated with nicotine content, construction of transgenic tobacco plants in which QPTase levels are lowered in the plant roots (compared to levels in wild-type plants) result in plants having reduced levels of nicotine in the leaves. Embodiments of the invention provide methods and nucleic acid constructs for producing such transgenic plants, as well as the transgenic plants themselves. Such methods include the expression of antisense NtQPT1 RNA, which lowers the amount of QPTase in tobacco roots.

Aspects of the present invention also concern sense and antisense recombinant DNA molecules encoding QPTase or QPTase antisense RNA molecules, and vectors comprising those recombinant DNA molecules, as well as transgenic plant cells and plants transformed with those DNA molecules and vectors. Transgenic tobacco cells and the plants described herein are characterized in that they have a reduced amount of nicotine and/or TSNA as compared to unmodified or control tobacco cells and plants.

Promoters to be linked to the antisense constructs of the present invention may be constitutively active promoters. Numerous constitutively active promoters which are operable in plants are available. A preferred example is the Cauliflower Mosaic Virus

(CaMV) 35S promoter which is expressed constitutively in most plant tissues. In the alternative, the promoter may be a root-specific promoter or root cortex specific promoter, as explained in greater detail below.

Antisense sequences have been expressed in transgenic tobacco plants utilizing the Cauliflower Mosaic Virus (CaMV) 35S promoter. See, e.g., Cornelissen *et al.*, "Both RNA Level and Translation Efficiency are Reduced by Anti-Sense RNA in Transgenic Tobacco", *Nucleic Acids Res.* 17, pp. 833-43 (1989); Rezaian *et al.*, "Anti-Sense RNAs of Cucumber Mosaic Virus in Transgenic Plants Assessed for Control of the Virus", *Plant Mol. Biol.* 11, pp. 463-71 (1988); Rodermel *et al.*, "Nuclear-Organelle Interactions: Nuclear Antisense Gene Inhibits Ribulose Bisphosphate Carboxylase Enzyme Levels in Transformed Tobacco Plants", *Cell* 55, pp. 673-81 (1988); Smith *et al.*, "Antisense RNA Inhibition of Polygalacturonase Gene Expression in Transgenic Tomatoes", *Nature* 334, pp. 724-26 (1988); Van der Krol *et al.*, "An Anti-Sense Chalcone Synthase Gene in Transgenic Plants Inhibits Flower Pigmentation", *Nature* 333, pp. 866-69 (1988).

Use of the CaMV 35S promoter for expression of antisense QPTase genes in the transformed tobacco cells and plants of this invention is preferred. Use of the CaMV promoter for expression of other recombinant genes in tobacco roots has been well described (Lam *et al.*, "Site-Specific Mutations Alter In Vitro Factor Binding and Change Promoter Expression Pattern in Transgenic Plants", *Proc. Nat. Acad Sci. USA* 86, pp. 7890-94 (1989); Poulsen *et al.* "Dissection of 5' Upstream Sequences for Selective Expression of the Nicotiana plumbaginifolia rbcS-8B Gene", *Mol. Gen. Genet.* 214, pp. 16-23 (1988).

Other promoters which are active only in root tissues (root specific promoters) are also particularly suited to the methods of the present invention. See, e.g., US Patent No. 5,459,252 to Conkling *et al.*; Yamamoto *et al.*, *Plant Cell*, 3:371 (1991). The *TobRD2* root-cortex specific promoter may also be utilized. See, eg., US Patent application SN 08/508,786, now allowed, to Conkling *et al*; PCT WO 9705261.

Some of the nucleic acids described herein may also be used in methods of sense co-suppression or RNAi-mediated suppression of nicotine production. Sense DNAs employed in these methods are preferably of a length sufficient to, when expressed in a plant cell, suppress the native expression of the plant QPTase protein as described herein in that plant cell. Such sense DNAs may be essentially an entire genomic or complementary DNA encoding the QPTase enzyme, or a fragment thereof, with such fragments typically being at least 15, 25, 27, 30, 35, 40, 45, 50, 60, 75, 100, 150, 250, 500, 750, nucleotides in

length. Methods of ascertaining the length of sense DNA that results in suppression of the expression of a native gene in a cell are available to those skilled in the art.

In an alternate embodiment, *Nicotiana* plant cells are transformed with a DNA construct containing a DNA segment encoding an enzymatic RNA molecule termed a "ribozyme", which enzymatic RNA molecule is directed against and cleaves the mRNA transcript of DNA encoding plant QPTase as described herein. Production of such an enzymatic RNA molecule in a plant cell and disruption of QPTase protein production reduces QPTase activity in plant cells in essentially the same manner as production of an antisense RNA molecule: that is, by disrupting translation of mRNA in the cell which produces the enzyme. The section below describes yet another method to decrease levels of specific enzymes involved in nicotine biosynthesis, using decoy nucleic acid fragments.

Molecular decoy technology to lower nicotine and/or TSNA levels

The use of nucleic acid-based decoy fragments to reduce gene expression is referred to as "molecular decoys". In a preferred example, the "decoy fragment" corresponds to promoter sequences upstream of the QPTase, to reduce QPTase expression.

In some embodiments, an isolated nucleic acid, or a fragment thereof consisting of at least 20-450 consecutive nucleotides desirably, at least 30-400 consecutive nucleotides preferably, 50-350 consecutive nucleotides more preferably, and 100-300 or 200-400 consecutive nucleotides most preferably, that is or contains at least one *cis*-acting regulatory element, which exists upstream of the plant QPTase and/or putrescine methyl transferase PMTase coding sequences (e.g., **SEQ. ID. No. 1**). Another example is the *Nic* gene product responsive element obtained from the sequence disclosed in U.S. Patent No. 5,459,252. In some embodiments, the *Nic* gene product responsive element resides between -1000 and -600 or -700 bp of the *NtQPT1* promoter. Accordingly, some embodiments involve a 300-400 nucleotide long fragment of the *NtQPT1* promoter that corresponds to the sequence of the *NtQPT1* promoter between -1000 and -600 or -700, as disclosed in U.S. Patent No. 5,459,252.

Thus, in several embodiments, the embodied nucleic acids have a structure that promotes an interaction with one or more transcription factors (e.g., *Nic1* and *Nic2)*, which are involved in initiating transcription of QPTase and/or PMTase. Accordingly, said nucleic acids are said to be or contain at least one transcription factor (e.g., *Nic1* and *Nic2)* binding sequences, which are also referred to as "*cis*-acting regulatory elements." By introducing multiple copies of these cis-acting regulatory elements (e.g., sequences that

interact with *Nic1* and/or *Nic2)* into a plant cell, the ability of the transcription factor to initiate transcription of the targeted gene (e.g., QPTase and/or PMTase genes) can be reduced or squelched.

By one approach, tobacco plants are transformed with an excess number of DNA sequences (*cis*-acting elements) from the promoters of genes encoding, but not limited to, QPTase and PMTase that are regulated in nicotine biosynthesis. These *cis*-acting elements are preferably integrated into the plant genome so as to allow for transfer to successive generations. Preferred approaches are provided in **Example 4** and **Example 5**. Typically, the *Nic1* and *Nic2* DNA-binding proteins that interact with these *cis*-acting DNA sequences are expressed at relatively low levels in the cell, thus the excess of transgenic *cis*-acting elements will compete with the endogenous elements associated with the genes encoding, but not limited to, QPTase and PMTase for available *Nic1* and *Nic2* Accordingly, these *cis*-acting DNA sequences (and those of other *cis*-acting elements) are referred to herein as "decoys" or "molecular decoys". The competition decreases occupancy of *trans*-acting DNA-binding proteins on their cognate *cis*-acting elements, thereby down-regulating the synthesis of nicotine biosynthesis enzymes.

Embodiments of the present invention also provide DNA molecules of *cis*-acting elements of QPTase or PMTase, and vectors comprising those DNA molecules, as well as transgenic plant cells and plants transformed with those DNA molecules and vectors. Transgenic tobacco cells and plants of this invention are characterized by lower nicotine content than untransformed control tobacco cells and plants.

Any of a variety of *cis*-acting elements can be used in carrying out the molecular decoy methods, depending upon the particular application. Examples of *cis*-acting elements (and corresponding transcription factors) that may be used, alone or in combination with one another, which may be used in embodiments of the present invention include, but are not limited to, AS-1 and ASF-1 (see U.S. Patents 4,990,607 and 5,223,419), the AATT repeat element and PABF (see U.S. Patents 5,834,236 and 6,191,258), a wounding-responsive *cis*-acting element from potato (Siebert et al., *Plant Cell* 1:961-8 (1989)), an embryo-specific *cis*-acting element from bean (Bustos et al, *Plant Cell* 1:839-853 (1989)), a root-specific *cis*-acting element from the tobacco RB7 promoter (US patent 5,459,252 and Yamamoto et al., *Plant Cell* 3:371-382 (1991)), a positive poly(dA-dT) regulatory element and binding protein and negative CCCAA repeat element and binding protein (Wang et al., *Mol. Cell Biol.* 12:3399-3406 (1992)), a root-tip regulatory element from the tobacco phytochrome A1 promoter of tobacco (Adam et al.,

Plant Mol Biol 29:983-993 (1995)), an anaerobiosis-responsive element from the maize glyceraldehyde-3-phosphate dehydrogenase 4 gene (Geffers et al., *Plant Mol Biol* 43:11-21 (2000)), and a seed-specific regulatory region from an *Arabidopsis oleosin* gene (see US patent 5,792,922).

The status of the art is such that large databases list identified cis-acting regulatory regions (e.g., Plant Cis-acting Regulatory elements, "PLACE", with about 1,340 entries, and Plant Cis-acting Regulatory Elements "PlantCARE", which lists about 159 plant promoters. The listed cis-acting regulatory elements in these databases and the cis-acting regulatory elements that are provided in Raumbauts et al., *Nucleic acids Research* 27:295-296 (1999), and Higo et al., *Nucleic acids Research* 27:297-300 (1999) can be used with embodiments of the invention. The section below describes general methods for transformation of tobacco plants with modified sequences to create tobacco plants with low nicotine and/or TSNA levels.

Transgenic Plant Cells and Plants

DNA sequences provided herein can be transformed into a variety of host cells. A variety of suitable host cells, having desirable growth and handling properties, are readily available in the art. As used herein, a "native DNA sequence" or "natural DNA sequence" means a DNA sequence which can be isolated from non-transgenic cells or tissue. Native DNA sequences are those which have not been artificially altered, such as by site-directed mutagenesis. Once native DNA sequences are identified, DNA molecules having native DNA sequences may be chemically synthesized or produced using recombinant DNA procedures as are known in the art. A native plant DNA sequence typically can be isolated from non-transgenic plant cells or tissue.

DNA constructs, or "transcription cassettes," of the present invention may include, 5' to 3' in the direction of transcription, a promoter as discussed herein, a DNA sequence as discussed herein operatively associated with the promoter, and, optionally, a termination sequence including stop signal for RNA polymerase and a polyadenylation signal for polyadenylase. The term "operatively associated," as used herein, refers to DNA sequences on a single DNA molecule which are associated so that the function of one is affected by the other. Thus, a promoter is operatively associated with a DNA when it is capable of affecting the transcription of that DNA (i.e., the DNA is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the DNA, which is in turn said to be "downstream" from the promoter.

In embodiments of the invention wherein a termination signal is used, any suitable termination signal may be employed in carrying out the present invention, examples thereof including, but not limited to, the nopaline synthase (*nos*) terminator, the octopine synthase (*ocs*) terminator, the CaMV terminator, or native termination signals derived from the same gene as the transcriptional initiation region or derived from a different gene. See, e.g., Rezian *et al.* (1988) supra, and Rodermel *et al.* (1988), supra. Alternatively, if nicotine levels are decreased by molecular decoy technology rather than by antisense or other methods, the molecular decoy fragments, with or without additional sequences, may be provided to the plant cell by any means. For example, the molecular decoy fragment may have an accompanying gene encoding a selectable marker, other suitable genes, or may be present as part of a plasmid vector. The molecular decoy fragment may consist of a single or double stranded DNA or RNA molecule. The molecular decoy may be integrated into the genome or may exist freely in the cell.

The transcription cassette may be provided in a DNA construct that also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coli,* such as ColEl, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there will frequently be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in a eukaryotic host, particularly the plant host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; may provide complementation, by imparting prototrophy to an auxotrophic host; or may provide a visible phenotype through the production of a novel compound in the plant.

The various fragments comprising the various constructs, transcription cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature as exemplified by J. Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory).

Vectors which may be used to transform plant tissue with nucleic acid constructs of the present invention include both *Agrobacterium* vectors and ballistic vectors, as well as vectors suitable for DNA.-mediated transformation. The term 'promoter' refers to a region of a DNA sequence that incorporates the necessary signals for the efficient expression of a coding sequence. This may include sequences to which an RNA polymerase binds but is not limited to such sequences and may include regions to which other regulatory proteins bind together with regions involved in the control of protein translation and may include coding sequences.

The QPTase recombinant DNA molecules and vectors used to produce the transformed tobacco cells and plants of this invention may further comprise a dominant selectable marker gene. Suitable dominant selectable markers for use in tobacco include, inter alia, antibiotic resistance genes encoding neomycin phosphotransferase (NPTII), and hygromycin phosphotransferase (HPT). Other well-known selectable markers that are suitable for use in tobacco include a mutant dihydrofolate reductase gene that encodes methotrexate-resistant dihydrofolate reductase. DNA vectors containing suitable antibiotic resistance genes, and the corresponding antibiotics, are commercially available.

Transformed tobacco cells are selected out of the surrounding population of non-transformed cells by placing the mixed population of cells into a culture medium containing an appropriate concentration of the antibiotic (or other compound normally toxic to tobacco cells) against which the chosen dominant selectable marker gene product confers resistance. Thus, only those tobacco cells that have been transformed will survive and multiply. Additionally, the positive selection techniques described by Jefferson (e.g., WO 00055333; WO 09913085; U.S. Pat. Nos. 5599670; 5432081; and 5268463) can be used.

Methods of making recombinant plants of the present invention, in general, involve first providing a plant cell capable of regeneration (the plant cell typically residing in a tissue capable of regeneration). The plant cell is then transformed with a DNA construct comprising a transcription cassette of the present invention (as described herein) and a recombinant plant is regenerated from the transformed plant cell. As explained below, the transforming step is carried out by techniques as are known in the art, including but not limited to bombarding the plant cell with microparticles, carrying the transcription cassette, infecting the cell with an *Agrobacterium tumefaciens* containing a Ti plasmid carrying the transcription cassette or any other technique suitable for the production of a transgenic plant.

Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known. For example, U.S. Patent No. 4,459,355 discloses a method for transforming susceptible plants, including dicots, with an *Agrobacterium* strain containing the Ti plasmid. The transformation of woody plants with an *Agrobacterium* vector is disclosed in U.S. Patent No. 4,795,855. Further, U.S. Patent No. 4,940,838 to Schilperoort *et al.* discloses a binary *Agrobacterium* vector (i.e., one in which the *Agrobacterium* contains one plasmid having the vir region of a Ti plasmid but no T region, and a second plasmid having a T region but no vir region) useful in carrying out the present invention.

Microparticles carrying a DNA construct of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention. The microparticle is propelled into a plant cell to produce a transformed plant cell, and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050, and in Christou *et al.*, U.S. Patent No. 5,015,580. When using ballistic transformation procedures, the transcription cassette may be incorporated into a plasmid capable of replicating in or integrating into the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts; and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art. Fusion of tobacco protoplasts with DNA-containing liposomes or via electroporation is known in the art. (Shillito *et al.*, "Direct Gene Transfer to Protoplasts of Dicotyledonous and Monocotyledonous Plants by a Number of Methods, Including Electroporation", *Methods Enzymol.* 153, pp. 313-36 (1987)).

As used herein, transformation refers to the introduction of exogenous DNA into cells, so as to produce transgenic cells stably transformed with the exogenous DNA. Transformed cells are induced to regenerate intact tobacco plants through application of tobacco cell and tissue culture techniques that are well known in the art. The method of plant regeneration is chosen so as to be compatible with the method of transformation. The stable presence and the orientation of the QPTase sequence in transgenic tobacco plants can

be verified by Mendelian inheritance of the QPTase sequence, as revealed by standard methods of DNA analysis applied to progeny resulting from controlled crosses. After regeneration of transgenic tobacco plants from transformed cells, the introduced DNA sequence is readily transferred to other tobacco varieties through conventional plant breeding practices and without undue experimentation.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the transcription cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or TI) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as nptII) can be associated with the transcription cassette to assist in breeding.

As used herein, a crop comprises a plurality of plants of the present invention, and of the same genus, planted together in an agricultural field. Thus, the present invention provides a method of producing a crop of plants having lowered QPTase or PMTase activity and thus having decreased nicotine and/or TSNA levels, as compared to a similar crop of non-transformed plants of the same species and variety.

Levels of nicotine in the transgenic tobacco plants of the present invention can be detected by standard nicotine assays. Transformed plants in which the level of QPTase or

PMTase is reduced compared to untransformed control plants will accordingly have a reduced nicotine level compared to the control.

The modified tobacco plants described herein are suitable for conventional growing and harvesting techniques (e.g. topping or no topping, bagging the flowers or not bagging the flowers, cultivation in manure rich soil or without manure). The harvested tobacco leaves and stems are suitable for conventional methods of processing such as curing and blending. The modified tobacco is suitable for use in any traditional tobacco product including, but not limited to, pipe, cigar and cigarette tobacco, and chewing tobacco in any form including leaf tobacco, shredded tobacco, or cut tobacco. The section below describes typical curing methods which may be used to prepare the tobacco once it is harvested.

Curing

The curing process, which typically lasts about 1 week, brings out the flavor and aroma of tobacco. Several methods for curing tobacco may be used, and indeed many methods have been previously disclosed. For example, U.S. Pat. Nos. 4,499,911 to Johnson; 5,685,710 to Martinez Sagrera; 3,905,123 to Fowler; 3,840,025 to Fowler; and 4,192,323 to Horne describe aspects of the tobacco curing process which may be used for some embodiments of the present invention. Conventionally, "sticks" that are loaded with tobacco are placed into bulk containers and placed into closed buildings having a heat source known as a curing barn. A flue is often used to control the smoke (thus earning the term "flue-cured"). The method of curing will depend, in some cases, on the type of tobacco-use cessation product desired, (i.e., snuff, cigarettes, or pipe tobacco may preferably utilize different curing methods) and preferred methods may vary from region to region and in different countries. In some approaches, the stems and midveins of the leaf are removed from the leaves prior to curing to yield a high quality, low nitrosamine tobacco product.

"Flue curing" is a popular method for curing tobacco in Virginia, North Carolina, and the Coastal Plains regions of the United States. This method is used mainly in the manufacture of cigarettes. Flue curing requires a closed building equipped with a system of ventilation and a source of heat. The heating can be direct or indirect (e.g., radiant heat). When heat and humidity are controlled, leaf color changes, moisture is quickly removed, and the leaf and stems dry. Careful monitoring of the heating and humidity can reduce the accumulation of nitrosamines.

Another curing method is termed "air curing". In this method, an open framework is prepared in which sticks of leaves (or whole plants) are hung so as to be protected from both wind and sun. Leaf color changes from green to yellow, as leaves and stems dry slowly.

"Fire curing" employs an enclosed barn similar to that used for flue curing. The tobacco is hung over low temperature fire so that the leaves cure in a smoke-laden atmosphere. This process uses lower temperatures, so the process may take up to a month, in contrast to flue curing, which takes about 6 to 8 days.

A further curing method, termed "sun curing" is the drying of uncovered sticks or strings of tobacco leaves in the sun. The best known sun-cured tobaccos are the so-called oriental tobaccos of Turkey, Greece, Yugoslavia, and nearby countries.

The curing process, and most particularly the flue-curing process, is generally divided into the following four stages:

A) Firing Up: During this step, the tobacco leaves turn bright lemon-orange in color. This is achieved by a gradual increase in temperature.

B) Leaf Yellowing: In this step any moisture is removed. This creates the "yellowing" of the tobacco. It also prepares the tobacco for drying in the next step.

C) Leaf Drying: Leaf drying, an important step in the curing process, requires much time for the tobacco to dry properly. Additionally, air flow is increased in this step to facilitate the drying process.

D) Stem Drying: The drying process continues, as the stem of the tobacco leaf becomes dried.

The cured tobacco may then be blended with other tobaccos or other materials to create the product to be used for the tobacco-use cessation method. The section below describes typical methods of blending and preparing the tobacco product.

Tobacco blending

It may be desirable to blend tobacco of varying nicotine levels to create the cessation product having the desired level of nicotine. This blending process is typically

performed after the curing process, and may be performed by conventional methods. Preferred tobacco blending approaches are provided in **Examples 6** and **7**. In some embodiments, blending of the transgenic tobacco is conducted to prepare the tobacco so that it will contain specific amounts of nicotine and/or TSNA in specific products. Preferably, the blending is conducted so that tobacco products of varying amounts of nicotine and/or TSNAs are made in specific products.

A mixture that contains different types of tobacco is desirably substantially homogeneous throughout in order to avoid undesirable fluctuations in taste or nicotine levels. Typically, tobacco to be blended may have a moisture content between 30 and 75%. As an example, the tobacco is first cut or shredded to a suitable size, then mixed in a mixing device, such as a rotating drum or a blending box. One such known mixing device is a tumbling apparatus that typically comprises a rotating housing enclosing mixing paddles which are attached to and, therefore, rotate with the housing to stir the tobacco components together in a tumbling action as the drum turns.

After the desired tobaccos are thoroughly mixed, the resulting tobacco blend is removed from the mixing apparatus and bulked to provide a continuous, generally uniform quantity of the tobacco blend. The tobacco is then allowed to remain relatively undisturbed (termed the "bulking step") for the required period of time before subsequent operations are performed. The bulking step typically takes 30 minutes or less, and may be carried out on a conveyor belt. The conveyor belt allows the blended tobacco to remain in bulk form in an undisturbed condition while it is continuously moving the tobacco blend through the process from the mixing stage to the expansion stage.

The tobacco blend is typically expanded by the application of steam. The tobacco mixture is typically subjected to at least 0.25 pounds of saturated steam at atmospheric conditions per pound of blended tobacco for at least 10 seconds to provide an increase in moisture of at least 2 weight percent to the tobacco blend. After the tobacco blend has been expanded, it is dried. A typical drying apparatus uses heated air or superheated steam to dry the tobacco as the tobacco is conveyed by the heated air or steam stream through a drying chamber or series of drying chambers. Generally, the wet bulb temperature of the drying air may be from about 150 degrees F. to about 211 degrees F. The tobacco blend is typically dried to a moisture content of from about 60 percent to about 5 percent. The dried, expanded tobacco blend is then in a suitable mode to be processed into the tobacco-use cessation product as described below.

Nicotine reduction and/or tobacco-use cessation programs methods

It is also contemplated that the low nicotine and/or TSNA tobacco described herein can be processed and blended with conventional tobacco so as to create a wide-range of tobacco products with varying amounts of nicotine and/or nitrosamines. These blended tobacco products can be used in nicotine reduction and/or tobacco-use cessation programs so as to move a consumer from a high nicotine and TSNA product to a low nicotine and TSNA product.

In some embodiments of the invention, a stepwise nicotine reduction and/or tobacco-use cessation program can be established using the low nicotine, low TSNA products described above. As an example, the program participant initially determines his or her current level of nicotine intake. The program participant then begins the program at step 1, with a tobacco product having a reduced amount of nicotine, as compared to the tobacco product that was used prior to beginning the program. After a period of time, the program participant proceeds to step 2, using a tobacco product with less nicotine than the products used in step 1. The program participant, after another period of time, reaches step 3, wherein the program participant begins using a tobacco product with less nicotine than the products in step 2, and so on. Ultimately, the program participant uses a tobacco product having an amount of nicotine that is less than that which is sufficient to become addictive or to maintain an addiction. Thus, the nicotine reduction and/or tobacco-use cessation program limits the exposure of a program participant to nicotine and, concomitantly, the harmful effect of nicotine yet retains the secondary factors of addiction, including but not limited to, smoke intake, oral fixation, and taste.

For example, a smoker can begin the program smoking blended cigarettes having 5mg of nicotine and 1.5μg of nitrosamine, gradually move to smoking cigarettes with 3mg of nicotine and 1μg of nitrosamine, followed by cigarettes having 1mg nicotine and 0.5μg nitrosamine, followed by cigarettes having 0.5mg nicotine and 0.25μg nitrosamine, followed by cigarettes having less than 0.1mg nicotine and less than 0.1μg TSNA until the consumer decides to smoke only the cigarettes having virtually no nicotine and nitrosamines or quitting smoking altogether. Preferably, a three-step program is followed whereby at step 1, cigarettes containing 0.6mg nicotine and less than 2μg/g TSNA are used; at step 2, cigarettes containing 0.3mg nicotine and less than 1μg/g TSNA are used; and at step 3, cigarettes containing less than 0.1mg nicotine and less than 0.7μg/g TSNA are used. More preferably, a three-step program is followed whereby at step 1, cigarettes

containing 0.6mg nicotine and less than 2μg/g TSNA are used; at step 2, cigarettes containing 0.3mg nicotine and less than 1μg/g TSNA are used; and at step 3, cigarettes containing less than 0.05mg nicotine and less than 0.7μg/g TSNA are used. Accordingly, the blended cigarettes described herein provide the basis for an approach to reduce the carcinogenic potential in a human in a step-wise fashion.

The methods described herein facilitate tobacco-use cessation by allowing the individual to retain the secondary factors of addiction such as smoke intake, oral fixation, and taste, while gradually reducing the addictive nicotine levels consumed. Eventually, complete cessation is made possible because the presence of addiction for nicotine is gradually decreased while the individual is allowed to maintain dependence on the secondary factors, above.

Embodiments, for example, include stepwise blends of tobacco products, which are prepared with a variety of amounts of nicotine. These stepwise blends are made to have reduced levels of TSNAs and varying amounts of nicotine. As an example, cigarettes may contain, for example, 5 mg, 4, 3, 2, 1, 0.5, 0.1, or 0 mg of nicotine per cigarette. More preferably, blended cigarettes contain less than 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, and 0.6% nicotine.

In another aspect of the invention, the cigarettes of varying levels of nicotine are packaged to clearly indicate the level of nicotine present, and marketed as a smoking cessation program. A preferred approach to produce a product for nicotine reduction and/or tobacco-use cessation program is provided in **Example 8**. Individuals may wish to step up the program by skipping gradation levels of nicotine per cigarette or staying at certain steps until ready to proceed to the next level. Significantly, aspects of the invention allow a consumer to individually select the amount of nicotine that is ingested by selection of a particular tobacco product described herein. Furthermore, because the secondary factors of addiction are maintained, dependence on nicotine can be reduced rapidly.

The nicotine reduction and/or tobacco-use cessation program limits the exposure of a program participant to nicotine while retaining the secondary factors of addiction. These secondary factors include but are not limited to, smoke intake, oral fixation, and taste. Because the secondary factors are still present, the program participant may be more likely to be successful in the nicotine reduction and/or tobacco-use cessation program than in programs that rely on supplying the program participant with nicotine but remove the above-mentioned secondary factors. Ultimately, the program participant uses a tobacco

product having an amount of nicotine that is less than that which is sufficient to become addictive.

In another aspect of the invention, individuals would choose to obtain only cigarettes with less than 0.05 mg nicotine per cigarette. Some individuals, such as individuals needing to stop nicotine intake immediately (for example, individuals with medical conditions or individuals using drugs that interact with nicotine) may find this method useful. For some individuals, the mere presence of a cigarette in the mouth can be enough to ease withdrawal from nicotine addiction. Gradually, the addictive properties of smoking can decrease since there is no nicotine in the cigarettes. These individuals are then able to quit smoking entirely.

In another aspect of the invention, packs of cigarettes containing the gradations of nicotine levels are provided as a "smoking cessation kit." An individual who wishes to quit smoking can buy the entire kit of cigarettes at the beginning of the program. Thus any temptation that may occur while buying cigarettes at the cigarette counter is avoided. Thus, the success of this method may be more likely for some individuals. A preferred example of such a kit is provided in **Example 9**.

The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

EXAMPLE 1

Isolation and Sequencing

TobRD2 cDNA (Conkling *et. al.*, *Plant Phys.* 93, 1203 (1990)) encodes QPTase, which is predicted to be a cytosolic protein. Comparisons of the NtQPT1 amino acid sequence with the GenBank database revealed limited sequence similarity to certain bacterial and other proteins; quinolate phosphoribosyl transferase (QPTase) activity has been demonstrated for the *S. typhimurium*, *E. coli.* and *N. tabacum* genes. The NtQPT1 encoded QPTase has similarity to the deduced peptide fragment encoded by an *Arabidopsis* EST (expression sequence tag) sequence (Genbank Accession number F20096), which may represent part of an *Arabidopsis* QPTase gene.

EXAMPLE 2

Transformation of Tobacco Plants

DNA of the QPTase gene, in antisense orientation, is operably linked to a plant promoter (CaMV 35S or *TobRD2* root-cortex specific promoter) to produce two different

DNA cassettes: CaMV35S promoter/antisense QPTase-encoding gene and *TobRD2* promoter/antisense QPTase-encoding gene.

A wild-type tobacco line and a low-nicotine tobacco line are selected for transformation, e.g., wild-type Burley 21 tobacco (Nic1+/*Nic2*+) and homozygous *Nic1-/Nic2-* Burley 21. A plurality of tobacco plant cells from each line are transformed using each of the DNA cassettes. Transformation is conducted using an *Agrobacterium* vector, e.g., an *Agrobacterium*-binary vector carrying Ti-border sequences and the *nptII* gene (conferring resistance to kanamycin and under the control of the nos promoter (*nptII*)).

Transformed cells are selected and regenerated into transgenic tobacco plants called R_o. The R_o plants are grown to maturity and tested for levels of nicotine; a subset of the transformed tobacco plants exhibit significantly lower levels of nicotine compared to non-transformed control plants.

R_o plants are then selfed and the segregation of the transgene is analyzed in next generation, the R_1 progeny. R_1 progeny are grown to maturity and selfed; segregation of the transgene among R_2 progeny indicate which R_1 plants are homozygous for the transgene.

EXAMPLE 3

Tobacco having reduced nicotine levels

Tobacco of the variety Burley 21 LA was transformed with the binary *Agrobacterium* vector pYTY32 to produce a low nicotine tobacco variety, Vector 21-41. The binary vector pYTY32 carried the 2.0 kb *NtQPT1* root-cortex-specific promoter driving antisense expression of the *NtQPT1* cDNA and the nopaline synthase (*nos*) 3' termination sequences from *Agrobacterium tumefaciens* T-DNA. The selectable marker for this construct was neomycin phosphotransferase (*nptII*) from *E. coli* Tn5 which confers resistance to kanamycin, and the expression *nptII* was directed by the *nos* promoter from *Agrobacterium tumefaciens* T-DNA. Transformed cells, tissues, and seedlings were selected by their ability to grow on Murashige-Skoog (MS) medium containing 300 µg/ml kanamycin. Burley 21 LA is a variety of Burley 21 with substantially reduced levels of nicotine as compared with Burley 21 (*i.e.*, Burley 21 LA has 8% the nicotine levels of Burley 21, see Legg *et al.*, *Can J Genet Cytol*, 13:287-91 (1971); Legg *et al.*, *J Hered*, 60:213-17 (1969)).

One-hundred independent pYTY32 transformants of Burley 21 LA (T_0) were allowed to self. Progeny of the selfed plants (T_1) were germinated on medium containing kanamycin and the segregation of kanamycin resistance scored. T_1 progeny segregating 3:1 resulted from transformation at a single locus and were subjected to further analysis.

Nicotine levels of T_1 progeny segregating 3:1 were measured qualitatively using a micro-assay technique. Approximately ~200 mg fresh tobacco leaves were collected and ground in 1 ml extraction solution (Extraction solution: 1 ml Acetic acid in 100 ml H_2O). Homogenate was centrifuged for 5 min at 14,000 x g and supernatant removed to a clean tube, to which the following reagents were added: 100 µL NH_4OAC (5 g/100 ml H_2O + 50 µL Brij 35); 500 µL Cyanogen Bromide (Sigma C-6388, 0.5 g/100 ml H_2O + 50 µL Brij 35); 400 µL Aniline (0.3 ml buffered Aniline in 100 ml NH_4OAC + 50 µL Brij 35). A nicotine standard stock solution of 10 mg/ml in extraction solution was prepared and diluted to create a standard series for calibration. Absorbance at 460 nm was read and nicotine content of test samples were determined using the standard calibration curve.

T_1 progeny that had less than 10% of the nicotine levels of the Burley 21 LA parent were allowed to self to produce T_2 progeny. Homozygous T_2 progeny were identified by germinating seeds on medium containing kanamycin and selecting clones in which 100% of the progeny were resistant to kanamycin (*i.e.*, segregated 4:0; heterozygous progeny would segregate 3:1). Nicotine levels in homozygous and heterozygous T_2 progeny were qualitatively determined using the micro-assay and again showed levels less than 10% of the Burley 21 LA parent. Leaf samples of homozygous T_2 progeny were sent to the Southern Research and Testing Laboratory in Wilson, NC for quantitative analysis of nicotine levels using Gas Chromatography/Flame Ionization Detection (GC/FID). Homozygous T_2 progeny of transformant #41 gave the lowest nicotine levels (~70 ppm), and this transformant was designated as "Vector 21-41."

Vector 21-41 plants were allowed to self-cross, producing T_3 progeny. T_3 progeny were grown and nicotine levels assayed qualitatively and quantitatively. T_3 progeny were allowed to self-cross, producing T_4 progeny. Samples of the bulked seeds of the T_4 progeny were grown and nicotine levels tested.

In general, Vector 21-41 is similar to Burley 21 LA in all assessed characteristics, with the exception of alkaloid content and total reducing sugars (*e.g.*, nicotine and nor-nicotine). Vector 21-41 may be distinguished from the parent Burley 21 LA by its substantially reduced content of nicotine, nor-nicotine and total alkaloids. As shown

below, total alkaloid concentrations in Vector 21-41 are significantly reduced to approximately relative to the levels in the parent Burley 21 LA, and nicotine and nor-nicotine concentrations show dramatic reductions in Vector 21-41 as compared with Burley 21 LA. Vector 21-41 also has significantly higher levels of reducing sugars as compared with Burley 21 LA.

Field trials of Vector 21-41 T_4 progeny were performed at the Central Crops Research Station (Clayton, NC) and compared to the Burley 21 LA parent. The design was three treatments (Vector 21-41, a Burley 21 LA transformed line carrying only the *NtQPT1* promoter [Promoter-Control], and untransformed Burley 21 LA [Wild-type]), 15 replicates, 10 plants per replicate. The following agronomic traits were measured and compared: days from transplant to flowering; height at flowering; leaf number at flowering; yield; percent nicotine; percent nor-nicotine; percent total nitrogen; and percent reducing sugars.

Vector 21-41 was also grown on approximately 5000 acres by greater than 600 farmers in five states (Pennsylvania, Mississippi, Louisiana, Iowa, and Illinois). The US Department of Agriculture, Agriculture Marketing Service (USDA-AMS) quantified nicotine levels (expressed as percent nicotine per dry weight) using the FTC method of 2,701 samples taken from these farms. Nicotine levels ranged from 0.01% to 0.57%. The average percent nicotine level for all these samples was 0.09%, with the median of 0.07%. Burley tobacco cultivars typically have nicotine levels between 2% and 4% dry weight (Tso, T.C., 1972, *Physiology and Biochemistry of Tobacco Plants.* Dowden, Hutchinson, and Ross, Inc. Stroudsbury).

EXAMPLE 4

Regulation of *NtQPT1* Gene Expression Using Molecular Decoys

Nucleotide sequence located between –1000 and -600 or -700 bp of the *NtQPT1* promoter is inserted in tandem arrays into a plant-*Agrobacterium* shuttle vector and subsequently transformed into tobacco via methods known to one skilled in the art. Plants stably transformed with said vector are assessed for the level of expression of *NtQPT1* and for nicotine and/or TSNA content. These experiments demonstrate that tobacco transformed with molecular decoys that interact with *Nic* gene products exhibit a reduced level of expression of *NtQPT1*.

EXAMPLE 5

Tobacco Having Reduced Nicotine and/or TSNA Levels Generated Using Molecular Decoys

Multiple copies of an approximately 300 or 400 nucleotide long fragment of the *NtQPT1* promoter (e.g., including nucleotide sequence located between –1000 and -600 or -700 bp of the *NtQPT1* promoter), are affixed to microparticles (e.g., by precipitation) that are suitable for the ballistic transformation of a plant cell (e.g., 1 to 5 μm gold spheres). The microparticles are propelled into tobacco plant cells (e.g., Burley 21 LA) using any suitable ballistic cell transformation methodology, so as to produce transformed plant cells. Plants are then regenerated from the transformed plant cells. Burley 21 LA is a variety of Burley 21 with substantially reduced levels of nicotine as compared with Burley 21 (*i.e.*, Burley 21 LA has 8% the nicotine levels of Burley 21, see Legg *et al.*, *Can J Genet Cytol*, 13:287-91 (1971); Legg *et al.*, *J Hered*, 60:213-17 (1969))

Transformed cells, tissues, and seedlings are grown on Murashige-Skoog (MS) medium (with or without the selection compound, e.g., antibiotic, depending on whether a selectable marker was used. One-hundred independent transformants of Burley 21 LA (T_0) are allowed to self. Progeny of the selfed plants (T_1) are germinated. Nicotine levels of T_1 progeny are measured qualitatively using a micro-assay technique. Approximately 200 mg fresh tobacco leaves are collected and ground in 1 ml extraction solution. (Extraction solution: 1 ml Acetic acid in 100 ml H_2O) Homogenate is centrifuged for 5 min at 14,000 x g and supernatant removed to a clean tube, to which the following reagents are added: 100 μL NH_4OAC (5 g/100 ml H_2O + 50 μL Brij 35); 500 μL Cyanogen Bromide (Sigma C-6388, 0.5 g/100 ml H_2O + 50 μL Brij 35); 400 μL Aniline (0.3 ml buffered Aniline in 100 ml NH_4OAC + 50 μL Brij 35). A nicotine standard stock solution of 10 mg/ml in extraction solution is prepared and diluted to create a standard series for calibration. Absorbance at 460 nm is read and nicotine content of test samples are determined using the standard calibration curve.

T_1 progeny that have less than 10% of the nicotine levels of the Burley 21 LA parent are allowed to self to produce T_2 progeny. Homozygous T_2 progeny are then identified. Nicotine levels in homozygous and heterozygous T_2 progeny are also qualitatively determined using the micro-assay. Leaf samples of homozygous T_2 progeny can also be sent to the Southern Research and Testing Laboratory in Wilson, NC for quantitative analysis of nicotine levels using Gas Chromatography/Flame Ionization

Detection (GC/FID). Homozygous T_2 progeny will have nicotine levels that are substantially reduced as compared to the untransformed tobacco (e.g., ~70 ppm). Because the nicotine levels in such plants are substantially reduced, the TSNA levels in these plants is concomitantly reduced.

These experiments demonstrate that tobacco transformed with molecular decoys that interact with *Nic* gene products exhibit a reduced amount of nicotine and/or TSNA. Plants with multiple tandem insertions of the molecular decoy that have reduced *NtQPT1* expression and reduced nicotine/TSNA levels are used to generate commercially valuable tobacco products.

EXAMPLE 6

Low Nicotine and Nitrosamine blended Tobacco

The following example describes several ways to create tobacco products having specific amounts of nicotine and/or TSNAs through blending. Some blending approaches begin with tobacco prepared from varieties that have extremely low amounts of nicotine and/or TSNAs. By blending prepared tobacco from a low nicotine/TSNA variety (e.g., undetectable levels of nicotine and/or TSNAs) with a conventional tobacco (e.g., Burley, which has 30,000 parts per million (ppm) nicotine and 8,000 parts per billion (ppb) TSNA; Flue-Cured, which has 20,000 ppm nicotine and 300 ppb TSNA; and Oriental, which has 10,000 ppm nicotine and 100 ppb TSNA), tobacco products having virtually any desired amount of nicotine and/or TSNAs can be manufactured. Other approaches blend only low nicotine/TSNA tobaccos (e.g., genetically modified Burley, genetically modified Virginia flue, and genetically modified Oriental tobaccos that contain reduced amounts of nicotine and/or TSNAs). Tobacco products having various amounts of nicotine and/or TSNAs can be incorporated into tobacco-use cessation kits and programs to help tobacco users reduce or eliminate their dependence on nicotine and reduce the carcinogenic potential.

By one approach, a step 1 tobacco product is comprised of approximately 25% low nicotine/TSNA tobacco and 75% conventional tobacco; a step 2 tobacco product can be comprised of approximately 50% low nicotine/TSNA tobacco and 50% conventional tobacco; a step 3 tobacco product can be comprised of approximately 75% low nicotine/TSNA tobacco and 25% conventional tobacco; and a step 4 tobacco product can be comprised of approximately 100% low nicotine/TSNA tobacco and 0% conventional tobacco. A tobacco-use cessation kit can comprise an amount of tobacco product from

each of the aforementioned blends to satisfy a consumer for a single month program. That is, if the consumer is a one pack per day smoker, for example, a single month kit would provide 7 packs from each step, a total of 28 packs of cigarettes. Each tobacco-use cessation kit would include a set of instructions that specifically guide the consumer through the step-by-step process. Of course, tobacco products having specific amounts of nicotine and/or TSNAs would be made available in conveniently sized amounts (e.g., boxes of cigars, packs of cigarettes, tins of snuff, and pouches or twists of chew) so that consumers could select the amount of nicotine and/or TSNA they individually desire. There are many ways to obtain various low nicotine/low TSNA tobacco blends using the teachings described herein and the following is intended merely to guide one of skill in the art to one possible approach.

To obtain a step 1 tobacco product, which is a 25% low nicotine/TSNA blend, prepared tobacco from an approximately 0 ppm nicotine/TSNA tobacco can be mixed with conventional Burley, Flue-cured, or Oriental in a 25%/75% ratio respectively to obtain a Burly tobacco product having 22,500 ppm nicotine and 6,000 ppb TSNA, a Flue-cured product having 15,000 ppm nicotine and 225 ppb TSNA, and an Oriental product having 7,500 ppm nicotine and 75 ppb TSNA. Similarly, to obtain a step 2 product, which is 50% low nicotine/TSNA blend, prepared tobacco from an approximately 0 ppm nicotine/TSNA tobacco can be mixed with conventional Burley, Flue-cured, or Oriental in a 50%/50% ratio respectively to obtain a Burly tobacco product having 15,000 ppm nicotine and 4,000 ppb TSNA, a Flue-cured product having 10,000 ppm nicotine and 150 ppb TSNA, and an Oriental product having 5000 ppm nicotine and 50 ppb TSNA. Further, a step 3 product, which is a 75%/25% low nicotine/TSNA blend, prepared tobacco from an approximately 0 ppm nicotine/TSNA tobacco can be mixed with conventional Burley, Flue-cured, or Oriental in a 75%/25% ratio respectively to obtain a Burly tobacco product having 7,500 ppm nicotine and 2,000 ppb TSNA, a Flue-cured product having 5,000 ppm nicotine and 75 ppb TSNA, and an Oriental product having 2,500 ppm nicotine and 25 ppb TSNA.

It should be appreciated that tobacco products are often a blend of many different types of tobaccos, which were grown in many different parts of the world under various growing conditions. As a result, the amount of nicotine and TSNAs will differ from crop to crop. Nevertheless, by using conventional techniques one can easily determine an average amount of nicotine and TSNA per crop used to create a desired blend. By adjusting the amount of each type of tobacco that makes up the blend one of skill can balance the amount of nicotine and/or TSNA with other considerations such as appearance, flavor, and

smokability. In this manner, a variety of types of tobacco products having varying level of nicotine and/or nitrosamine, as well as, appearance, flavor and smokability can be created.

EXAMPLE 7

Low Nicotine and Nitrosamine blended Tobacco

By a preferred method, conventional Virginia flue tobacco was blended with genetically modified Burley (i.e., Burley containing a significantly reduced amount of nicotine and nitrosamine) to yield a blended tobacco that was incorporated into three levels of reduced nicotine cigarettes: a step 1 cigarette containing 0.6mg nicotine, a step 2 cigarette containing 0.3mg nicotine, and a step 3 cigarette containing less than 0.05mg nicotine. The amount of total TSNA was found to range between approximately 0.17μg/g - 0.6μg/g.

In some cigarettes, approximately, 28% of the blend was Virginia flue tobacco, approximately 29% of the blend was genetically modified (i.e., reduced nicotine Burley), approximately 14% of the blend was Oriental, approximately 17% of the blend was expanded flue-cured stem, and approximately 12% was standard commercial reconstituted tobacco. The amount of total TSNAs in cigarettes containing this blend was approximately 1.5μg/g.

EXAMPLE 8

Nicotine Reduction and/or Smoking Cessation Program Containing Low Nicotine And Nitrosamine Levels

The following example describes a nicotine reduction and/or smoking cessation program utilizing the low nicotine, low TSNA tobacco products of the present invention. The modified tobacco containing very low levels of TSNAs and essentially no nicotine is mixed with tobacco having a known amount of nicotine to create specific, stepwise levels of nicotine per cigarette. As an example, Virginia flue tobacco was blended with genetically modified Burley (i.e., Burley containing a significantly reduced amount of nicotine and nitrosamine) to yield a blended tobacco that was incorporated into three levels of reduced nicotine cigarettes: a step 1 cigarette containing 0.6mg nicotine, a step 2 cigarette containing 0.3mg nicotine, and a step 3 cigarette containing less than 0.05mg nicotine. The stepwise packs of cigarettes are clearly marked as to their nicotine content, and the step in the stepwise nicotine reduction program is also clearly marked on the

package. Each week, the user purchases packs containing cigarettes having the next lower level of nicotine, but limits himself to no more cigarettes per day than consumed previously. The user may define his/her own rate of nicotine reduction and/or smoking cessation according to individual needs by choosing a) the number of cigarettes smoked per day b) the starting nicotine levels c) the change in nicotine level per cigarette each week, and d) the final level of nicotine consumed per day. To keep better track of the program, the individual keeps a daily record of total nicotine intake, as well as the number of cigarettes consumed per day. Eventually, the individual will be consuming tobacco products with essentially no nicotine. Since the nicotine-free tobacco products of the final step are non-addictive, it should then be much easier to quit the use of the tobacco products altogether.

EXAMPLE 9

Nicotine Reduction and/or Smoking Cessation Kit Containing Packs Of Cigarettes With Low TSNA Levels And Stepwise Reductions In Nicotine Levels

Various nicotine reduction and/or smoking cessation kits are prepared, geared to heavy, medium, or light smokers. The kits provide all of the materials needed to quit smoking in either a two-week period (fast), a one-month period (medium) or in a two-month period (slow), depending on the kit. Each kit contains a set number of packs of cigarettes modified according the present invention, containing either step 1 cigarettes containing 0.6mg nicotine, step 2 cigarettes containing 0.3mg nicotine, and step 3 cigarettes containing less than 0.05mg nicotine. For example, 1 pack a day smokers would receive 7 packs of cigarettes, each pack containing the above amounts of nicotine per each cigarette. Several weeks worth of additional cigarettes containing less than 0.05 mg nicotine/cigarette would also be provided in the kit, to familiarize the consumer with smoking no nicotine cigarettes. The kit would also contain a diary for keeping track of daily nicotine intake, motivational literature to keep the individual interested in continuing the cessation program, health information on the benefits of smoking cessation, and web site addresses to find additional anti-smoking information, such as chat groups, meetings, newsletters, recent publications, and other pertinent links.

Although the invention has been described with reference to embodiments and examples, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.